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Beta-Galactosidase Activated Pro-Drugs

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13. ABSTRACT (Maximum 200 Words)  Gene therapy shows promise for treating prostate cancer and is being exploited in several clinical trials. A major hurdle is establishing a method of verifying transgene activity in situ. $\beta$ -galactosidase ( $\beta$ -gal) was historically the most popular reporter gene for molecular biology. I have introduced a novel concept for further exploration of gene therapy using $\beta$ -galactosidase to activate a broad-spectrum chemotherapeutic to assess the efficacy of the pro-drugs in vitro and explore growth delay in animal models. I also have developed a new $\beta$ -galactosidase molecular reporter for MRI spectra, which can be used to detection of lacZ gene expression in vivo.				
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## Table of Contents

Cover.....	1
SF 298 .....	2
Table of Contents .....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	6
Reportable Outcomes.....	13
Conclusions.....	14
Appendices.....	15

## Introduction

Prostate cancer is one of the most common malignant tumors with increasing incidence rates in the aging male, presenting a formidable public health problem. Gene-based therapy has been stimulated by remarkable progress in understanding molecular biology. Gene therapy holds great promise for the treatment of diverse diseases, The *lacZ* gene, encoding the enzyme  $\beta$ -galactosidase ( $\beta$ -gal), has historically been the most common reporter gene used in molecular biology, many chromogenic or fluorogenic substrates are well established, but they are generally limited to histology or *in vitro* assays. Our research team applied PFONPG for gene therapy and developed new NMR spectra reporter molecules to assess gene expression *in vivo*.

There are three specific aims in my proposal:

Specific aim 1 (Completed in 1<sup>st</sup> year)

Establish stable *lacZ/lacY* transfected CaP cell lines and screen the transfected cell colonies for high expression efficiency and growth capability. Months (1-12)( Finished)

Specific aim 2 (Completed in this year)

Evaluate cytotoxicity of pro-drug in transfected cells *in vitro*. (Months 12-18)

Specific aim 3 (Ongoing)

Evaluate cytotoxicity of pro-drug in transfected cells *in vivo*. (Months 18-24)

## Body

### Specific aim 1 (Completed in 1<sup>st</sup> year)

Establish stable *lacZ/lacY* transfected CaP cell lines and screen the transfected cell colonies for high expression efficiency and growth capability. Months (1-12)

### Specific aim 2(Completed in 2<sup>nd</sup> year)

Evaluate cytotoxicity of pro-drug in transfected cells *in vitro*. (Months 12-18)

Task 4. Evaluate growth of cells *in vitro* and *in vivo* (Months 9-12)

Task 5. Test synthetic phenyl galactoside substrates for  $\beta$ -galactosidase activity in transfected CaP cells in culture (Months 12-15)

Task 6. Perform feasibility experiments to assess the bystander effect of the mixture of transfected and non-transfected CaP cells *in vitro* (Months 15-18)

### Specific aim 3 (Ongoing)

Evaluate cytotoxicity of pro-drug in transfected cells *in vivo*. (Months 18-24)

Task 7. Examine the growth characteristic in each cell type implanted S.C *in vivo* (Months 18-20)

Task 8. Examine synthetic phenyl galactoside substrates for  $\beta$ -galactosidase activity and growth delay in implanted tumor *in vivo* (Months 18-22)

Task 9. Prepare manuscripts and reports (Months 22-24)

### Key Research Accomplishments

1. Growth Curve for PC3 WT and PC3/*lacZ* in vitro and in vivo
2. Treatment PC3 and PC3/*lacZ* cells implanted S.C tumor by PFONPG
3. Novel <sup>19</sup>F NMR Imaging molecular reporter

## Key Research Accomplishments

### Evaluate cytotoxicity of pro-drug in transfected cells *in vitro*. (Months 12-18)

#### Task 4. Evaluate PC3 and PC3/lacZ cell growth in *vitro* and *in vivo*

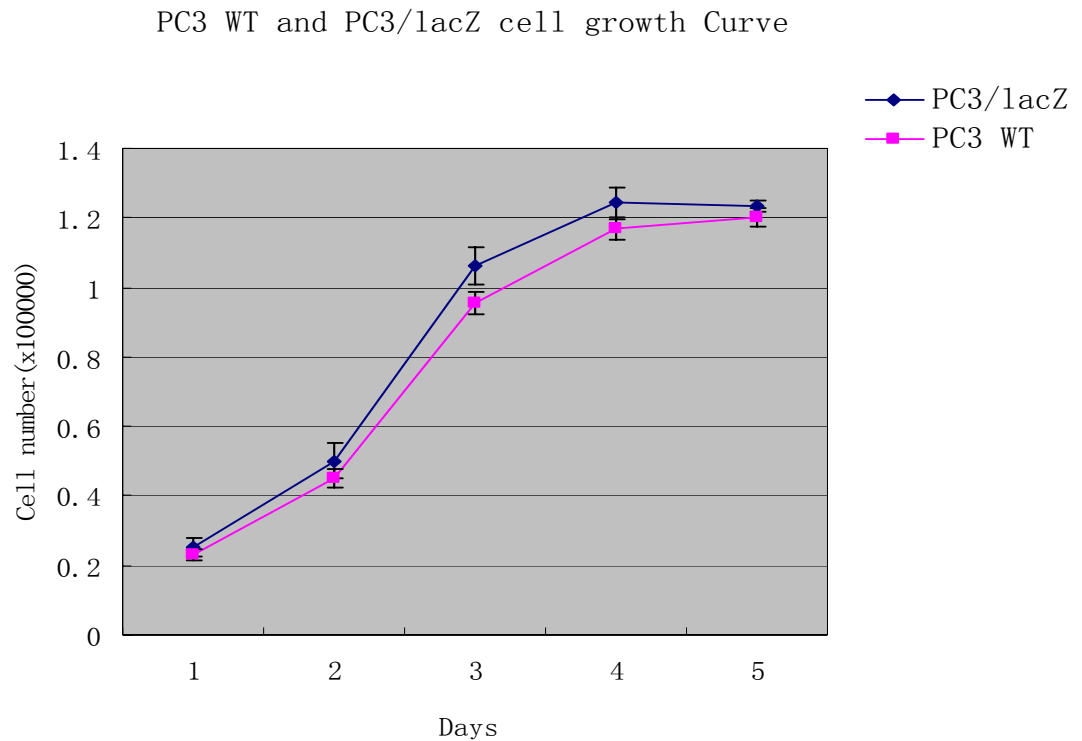


Fig. 1 - PC3 and PC3/lacZ cells growth curve

Growth curve: cells ( $3 \times 10^4$ ) were seeded into 24-well plates, the cell number in three to five flasks was determined at different times. PC3/lacZ expressing clones had slightly faster growth rate than the parental cells (WT).

For *in vivo* study,  $2 \times 10^6$  PC3 and  $1 \times 10^6$  PC3/lacZ cells were implanted subcutaneously into Nude mice and tumor growth was followed. Palpable tumors were formed 7-10 days after the injection. The lacZ over expressing cell lines formed tumors that grew much faster than the parental WT. (fig.2)

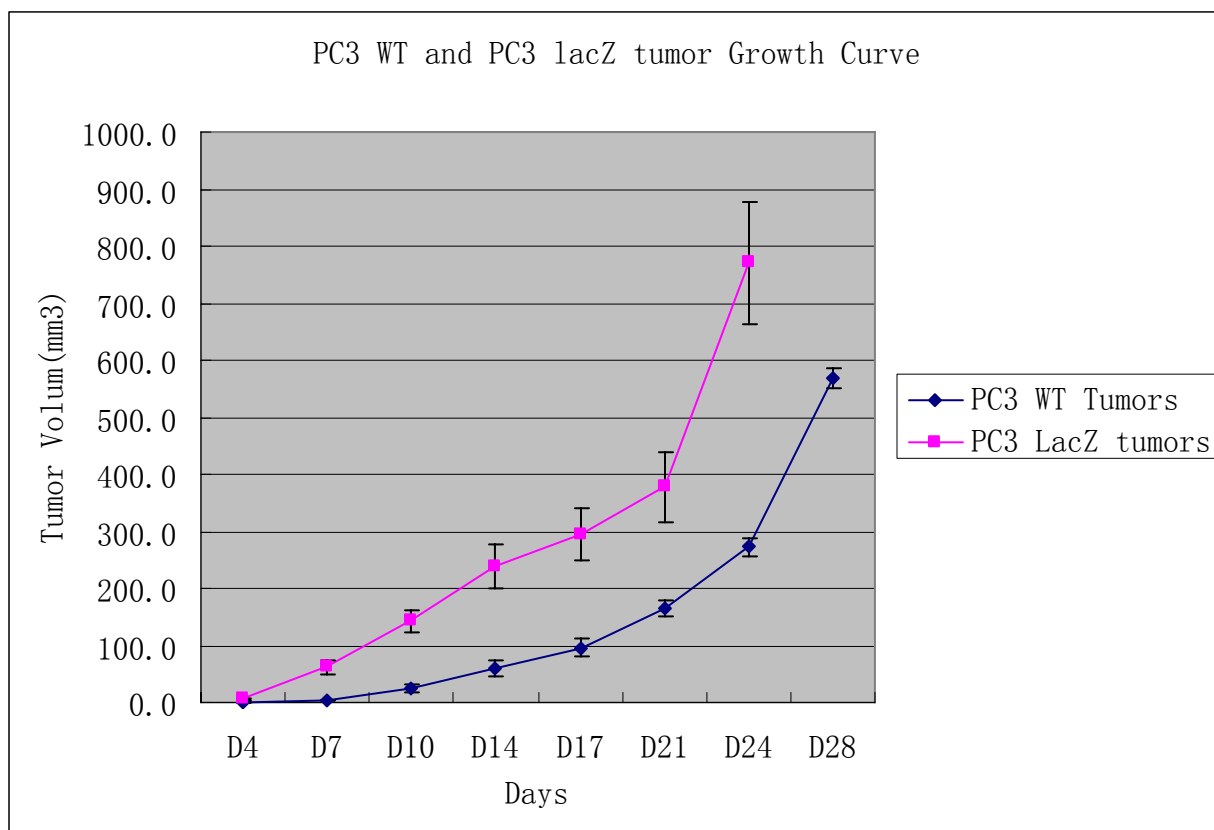


Fig. 2 - Growth Curve of PC3 and PC3/lacZ tumor in Nude mice (n=6)

**Task 5.** Test synthetic phenyl galactoside substrates for  $\beta$ -galactosidase activity in transfected CaP cells ( $1 \times 10^6$ )

ITEMS	PFONPG	OFPNPG	PCF3ONPG
PC3/lacZ	20.2 $\mu$ M/min	14.7 $\mu$ M/min	31.5 $\mu$ M/min
Chemical structure			

Fig. 3 - Hydrolytic rates of OFPNPG, PFONPG, PCF3ONPG with PC3/lacZ at 37°C in PBS.

I assessed several different synthetic phenyl galactoside substrates for  $\beta$ -galactosidase activity in PC3/lacZ cell and found PCF3ONPG has a highest hydrolytic rate.

**Task 6.** Perform feasibility experiments to assess the bystander effect of the mixture of transfected and non-transfected CaP cells *in vitro* (Months 15-18)

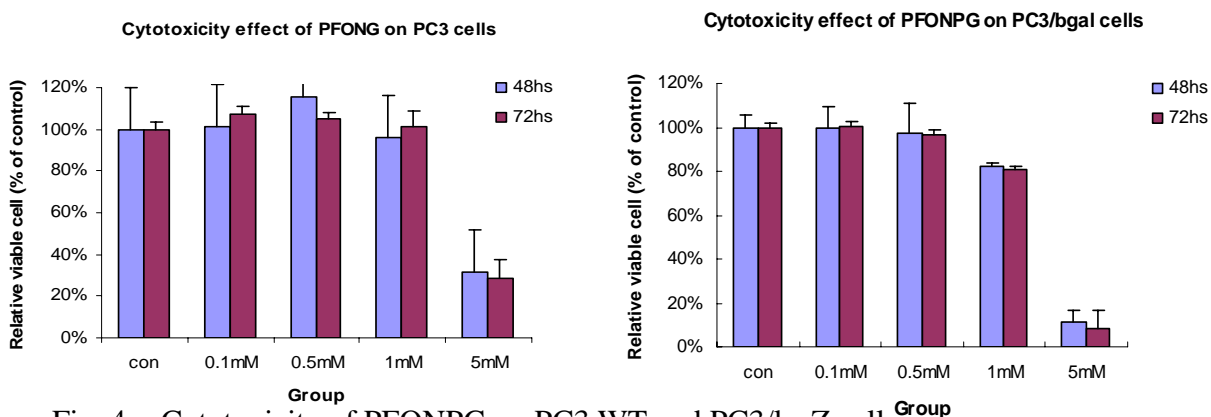


Fig. 4 - Cytotoxicity of PFONPG on PC3 WT and PC3/lacZ cells

To further investigate the effect of PFONPG on PC-3 and PC-3/lacZ cell growth inhibition,  $2 \times 10^4$  cell suspension was seeded in 24 well plate, after 24 hours, varies concentration of PFONPG were added. Cells were harvested of 48hrs and 72hrs and crystal violet methods were used to test the cell viability. Results show significant difference in 5mM between PC3 WT and PC3/lacZ cells. The aglycone PFONP is potentially cytolytic, being a close analog of the classic uncoupler DNP (dinitro phenol) and the observation of enzyme activated cytotoxicity for PFONPG in PC3/lacZ tranfected cells at high concentration about 5mM

### Specific aim 3 (Ongoing)

#### Evaluate cytotoxicity of pro-drug in transfected cells *in vivo*. (Months 18-24)

**Task 7.** Examine the growth characteristic in each cell type implanted S.C *in vivo* (Months 18-20)

#### 1. PFONPG treatment for PC3 and PC3/lacZ tumor xoengraft

(1) Low dose treatment: 40mg/kg PFONPG injected into two different group of mice twice weeks and continue treatment for 3 weeks. Measure tumors grow.

(2) High dose treatment: 200mg/kg PFONPG injected into two different groups' mice by IV twice weeks and continue treatment for 3 weeks, measure tumor grow.

(3) Saline as control: 100ul saline IV injected into control group. Same method is as treatment group mice. Every group n=6--8

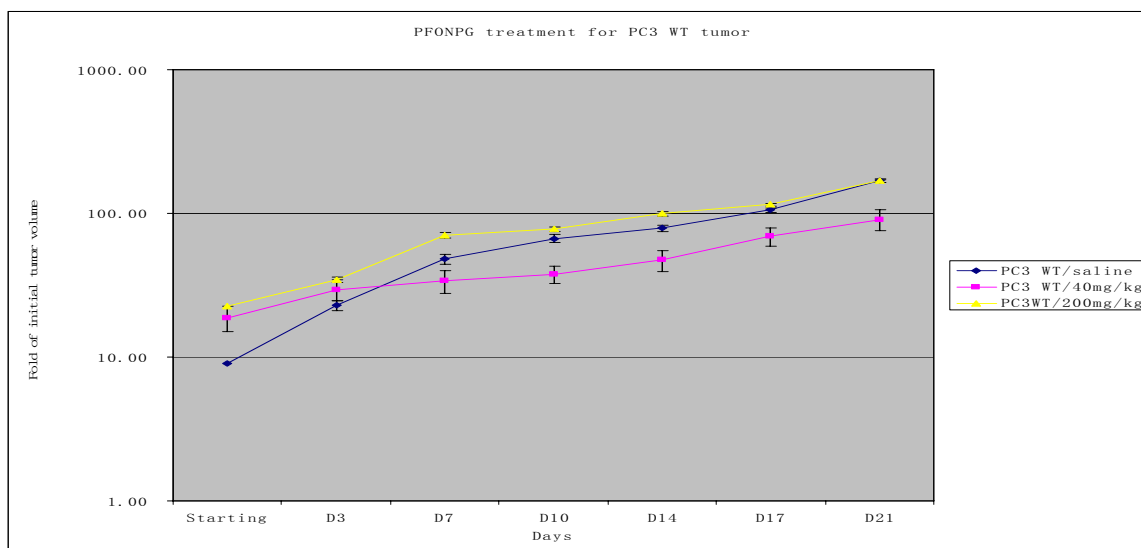


Fig. 5 - PFONPG treatment for PC3 WT tumor group

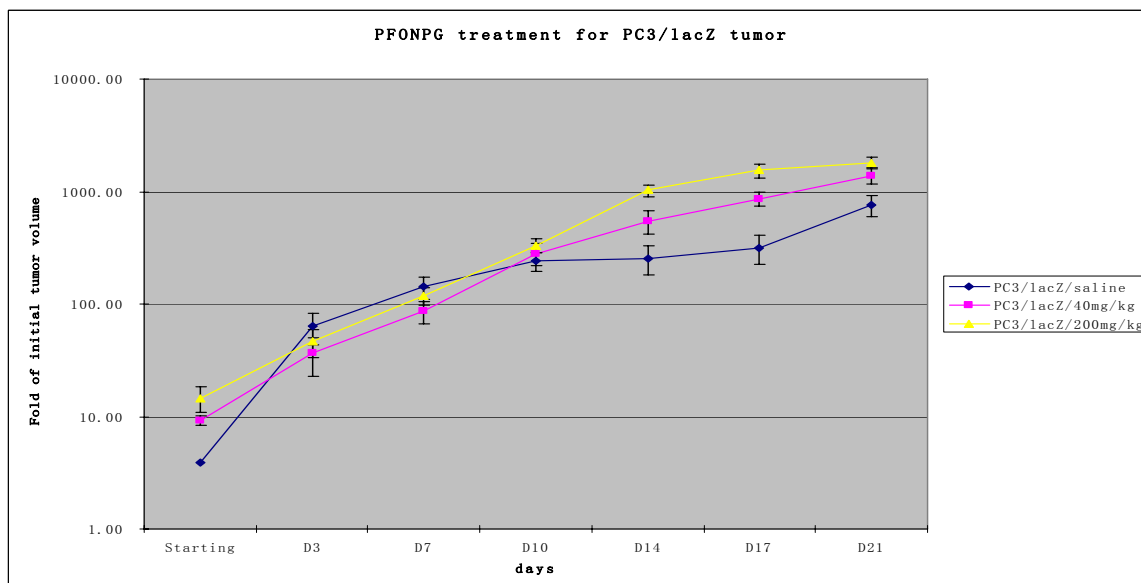


Fig. 6 - PFONPG treatment for PC3/lacZ tumor group

Comparing PFONPG treatment of the PC3 wild type to PC3/lacZ group, PFONPG appeared nontoxic. Other molecules are being synthesized.

## 2. Novel Agent for $^{19}\text{F}$ NMR

To develop enhanced reporter molecules, diverse substrates were synthesized and the activity of  $\beta$ -gal is shown for 3 representatives. 2-Fluoro-4-nitrophenol- $\beta$ -D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl- $\beta$ -D-galactopyranosides), which are highly responsive to the action of  $\beta$ -gal. OFPNPG has a single  $^{19}\text{F}$  peak at 55 ppm

relative to aqueous sodium trifluoroacetate (NaTFA). Upon cleavage by  $\beta$ -gal, the pH sensitive aglycone OFPNP is observed at a chemical shift of 59-61 ppm.

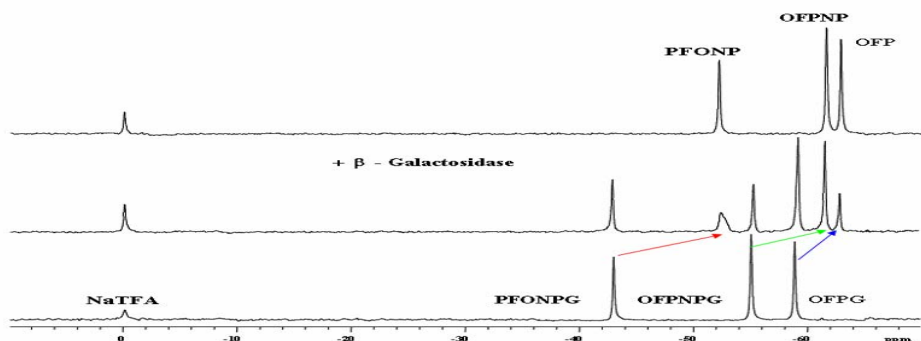


Fig. 7 -  $^{19}\text{F}$  NMR diverse substrates for  $\beta$ gal

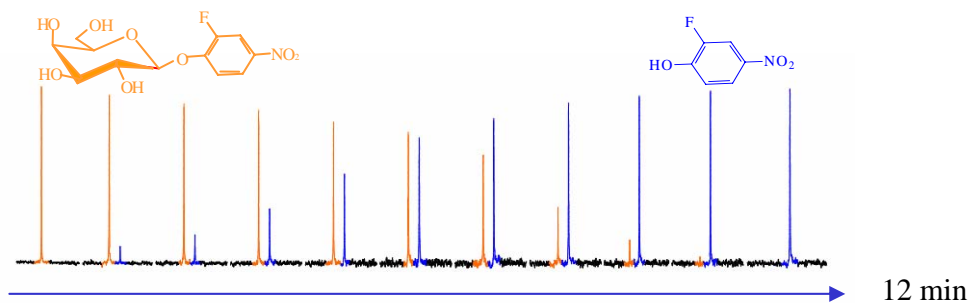


Fig. 8 - Hydrolytic kinetics of OFPNPG by PC3/LacZ prostate cancer cells

$5 \times 10^5$  PC3/lacZ cell in PBS buffer at  $37^\circ\text{C}$  (orange lines--signals of OFPNPG; blue lines--signals of product aglycone OFPNP). in PBS buffer at  $37^\circ\text{C}$

Over 12 min, subtract OFPNPG was converted to OFONP by  $\beta$ -gal enzyme activity in  $5 \times 10^5$  PC3/lacZ cell. Each spectrum was acquired in 1 min.

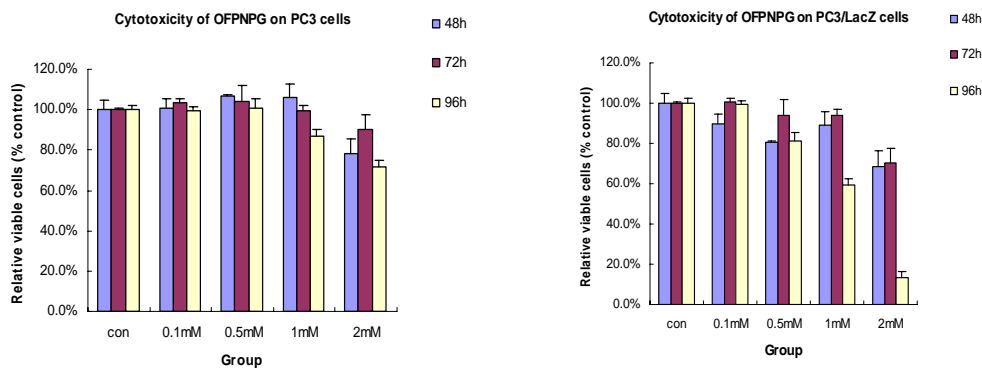


Fig. 9 - OFPNPG cytotoxicity in PC3 WT and PC3/lacZ cells

PC3 WT and PC3/lacZ cell viability for OFPNPG were detected by crystal violet methods; it is less toxic in PC3 WT cells than in PC3/lacZ cells. The graphs show that only OFPNPG concentration is about 2mM, PC3/lacZ cell had toxicity. This concentration is much higher than in vivo study.

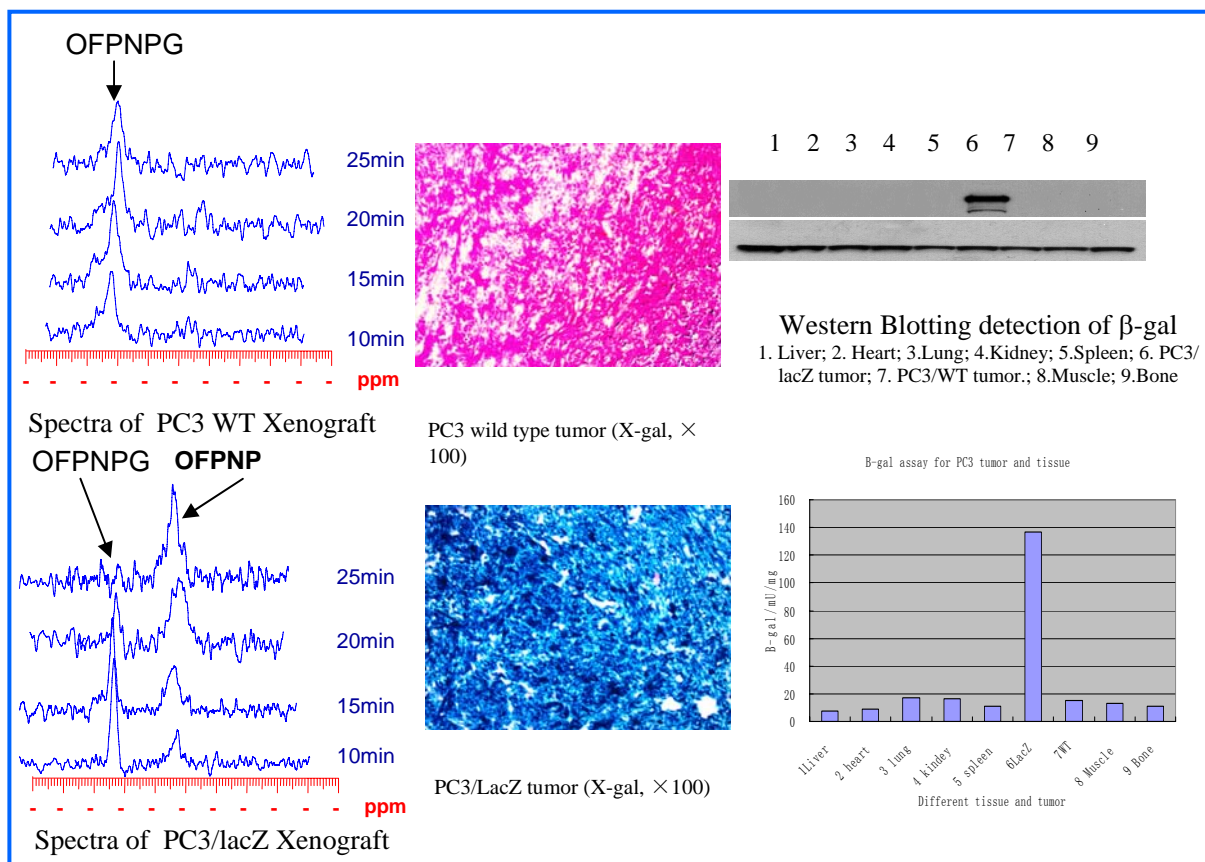


Fig. 10 - In vivo detection of lacZ gene expression in PC3 WT and PC3/lacZ tumors

PC3 wild type and PC3/ lacZ cells were implanted subcutaneously in flanks of nude mice to grow as solid tumors. When tumors reached about 200 mm<sup>3</sup> mice were anesthetized with isoflurane and OFPNPG (4 mg in 50 µl aqueous DMSO), containing sodium trifluoroacetate (Na-TFA, 10 mg/ml) as a standard, was injected intra tumorally (i.t.) using a fine 32G Hamilton syringe. <sup>19</sup>F spectroscopy was performed within 5 mins using a 4.7 T Varian scanner (188.2 MHz). Spectra were obtained with TR=1 s, na=128, SW=100 ppm and typically 30 Hz exponential line broadening was applied. Time course conversion of OFPNPG to OFPNP in PC3 and PC3/lacZ tumor <sup>19</sup>F NMR signal of OFPNPG was readily detected with a signal to noise ratio >10 in 5 mins following direct intra-tumoral injection. Over a period of 30min conversion of OFPNPG to product OFPNP was revealed by development of a new up field signal unequivocally demonstrating β-gal activity. Rapid conversion of OFPNPG to OFPNP was seen in PC3- lacZ tumors, while little or none was seen in WT tumors. These results provide further evidence for the utility of this class of substrate to generate Reporter Products for Magnetic resonance (RPMs) as a basis for molecular imaging to detect gene (viz. enzyme) activity.

After  $^{19}\text{F}$  NMR Spectroscopy of PC3 wt and PC3/lacZ Xenograft, the tumors were taken out and split in. One part was for histology and the other was for protein  $\beta$ -gal assay and Western blot. Comparing histology,  $\beta$ -gal assay and Western blot results, I found in PC3 WT has expression, and PC3/lacZ has really high expression for lacZ gene, also did not find  $\beta$ -gal expression in other organs.

## Conclusions

These results provide our first observations that the chemical shift response is sufficient to observe gal activity by  $^{19}\text{F}$  NMR in PC3 human prostate tumor xenografts in mice. This approach directly reveals  $\beta$ -gal activity, which could be used in tandem with therapeutic genes to monitor therapy. As gene therapy becomes a reality, the ability to detect transgene expression non-invasively will become increasingly important for treatment planning and optimization. OFPNPG is a promising lacZ gene reporter molecule for  $^{19}\text{F}$  MR spectroscopy. Meanwhile, we are also developing new generations of  $^{19}\text{F}$  NMR reporter designed to minimize toxicity.

## Reportable Outcomes

1. **L, Liu**, V. Kodibagkar, J. Yu and R. P. Mason, In vivo detection of *lacZ* gene expression in PC3 prostate xenograft tumor by  $^{19}\text{F}$  NMR, AACR 97<sup>th</sup> meeting, Washington DC, April, 2006
2. W. Cui, **L, Liu**, P. Peschke, U. Haberkorn, R. P. Mason, Transmembrane pH gradients in tumor cells: observations using  $^{19}\text{F}$  NMR of promising new reporter molecules, ISMRM 14<sup>th</sup> *Scientific Meeting in Seattle*, Washington, May, 2006
3. R. P. Mason, J. Yu, **L. Liu**, W. Cui and V. Kodibagkar, Novel Magnetic resonance Assays of Gene Imaging Constructs (MAGIC), Imaging in 2020, Jackson Hole, Wyoming, September 2005.
4. J. Yu, **L. Liu**, V. D. Kodibagkar, W. Cui, R. D. Gerard, R. P. Mason, Novel “Smart”  $^1\text{H}$  MRI Contrast Agents for Assessing *lacZ* Gene Expression” *ISMRM 13th Scientific Meeting in Miami Beach*, Florida, USA May 2005.
5. W. Cui, **L. Liu**, J. Yu, R. P. Mason, Detection of  $\beta$ -Galactosidase Activity in a Human Tumor Xenograft by  $^1\text{H}$  MRI in vivo Using S-Gal<sup>TM</sup>, *ISMRM 13th Scientific Meeting in Miami Beach*, Florida, USA May 2005.
6. V. Kodibagkar, J. Yu, **L. Liu**, S. Brown, H. P. Hetherington, R. D. Gerard, and R. P. Mason,  $^{19}\text{F}$  CSI of gene-reporter molecule OFPNPG”, *ISMRM 13th Scientific Meeting in Miami Beach*, Florida, USA May 2005.

## Conclusions

1. I developed a series of recombinant lacZ gene expression vectors
2. I screened several stable expression of lacZ cell lines, MAT-Lu/*lacZ* and PC3/*lacZ* cells
3. I confirmed high expression of *lacZ* gene in these cells by beta-gal activity assay X-staining and West blotting.
4. I got the growth curve PC3 and PC3/*lacZ* in vivo.
5. PFONPG treatment for PC3 and PC3/*lacZ* tumor
6. Develop novel <sup>19</sup>F NMR lacZ gene reporter molecules.
7. I am continuing to evaluate selective gene activated cytotoxicity.
8. I have also learnt techniques related to tumor implantation, treatment, small animal handling and MRI.

## Appendices

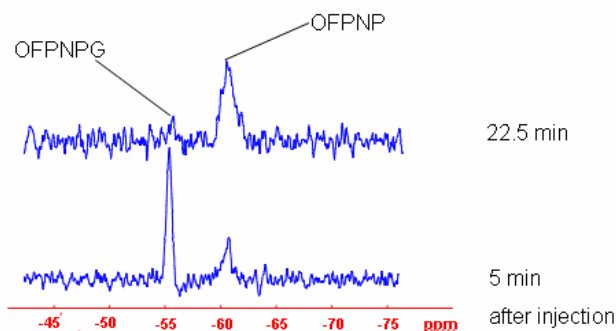
### Detection of *lacZ* Gene Expression in PC3 Prostate Xenograft by $^{19}\text{F}$ NMR

**Li Liu, Vikram Kodibagkar, Jianxin Yu, Ralph P. Mason**

Department of Radiology, The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75390-9058, USA. Email: [Li.Liu@utsouthwestern.edu](mailto:Li.Liu@utsouthwestern.edu)

Gene therapy shows promise for treating prostate cancer and has been successfully exploited in several clinical trials. A major hurdle is establishing a method of verifying transgene activity *in situ*.  $\beta$ -galactosidase ( $\beta$ -gal) has historically been the most popular reporter gene for molecular biology. We are designing non-invasive NMR approaches to reveal  $\beta$ -gal activity *in vivo*. 2-Fluoro-4-nitrophenol- $\beta$ -D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl- $\beta$ -D-galactopyranosides), which are highly responsive to the action of  $\beta$ -gal. OFPNPG has a single  $^{19}\text{F}$  peak at 55 ppm relative to aqueous sodium trifluoroacetate (NaTFA). Upon cleavage by  $\beta$ -gal, the pH sensitive aglycone OFPNP is observed at a chemical shift of 59-61 ppm. We now show the chemical shift response is sufficient to observe  $\beta$ -gal activity by NMR in PC3 human prostate tumor xenografts in mice.

PC3/LacZ tumor cells were generated by recombinant plasmid pHCMV/lacZ transfection and a high expressing clone selected. Cells were implanted in the flank of nude mice and allowed to grow to about  $1\text{cm}^3$ . When a solution of OFPNPG (4 mg in 50  $\mu\text{l}$  aqueous DMSO) was injected intra-tumorally,  $^{19}\text{F}$  NMR signal was readily detected at 4.7 T. Over a period of 30min conversion of OFPNPG to product OFPNP was observed unequivocally demonstrating  $\beta$ -gal activity (see spectra). Tumor and tissues were also examined by Western blots and  $\beta$ -gal assay for activity. High  $\beta$ -gal activity was found in the tumor, with minimal activity in normal tissues. This approach directly reveals  $\beta$ -gal activity, which could be used in tandem with therapeutic genes to monitor therapy. As gene therapy becomes a reality, the ability to detect transgenic expression non-invasively will become increasingly important for treatment planning and optimization. The prototype gene reporter molecule (OFPNPG) releases a potentially toxic product (fluronitrophenol) and we are also exploring whether this can serve as the basis for broad-spectrum chemotherapy. Meanwhile, we are also developing new generations of  $^{19}\text{F}$  NMR reporter designed to minimize toxicity.



**Keywords:**  $\beta$ -galactosidase,  $^{19}\text{F}$  NMR, lacZ gene, prostate cancer, gene therapy  
Supported by DOD PC031075 (LL), NCI pre-ICMIC CA86354 and P41-RR02584  
The 97<sup>th</sup> AACR meeting, Washington DC, April, 2006

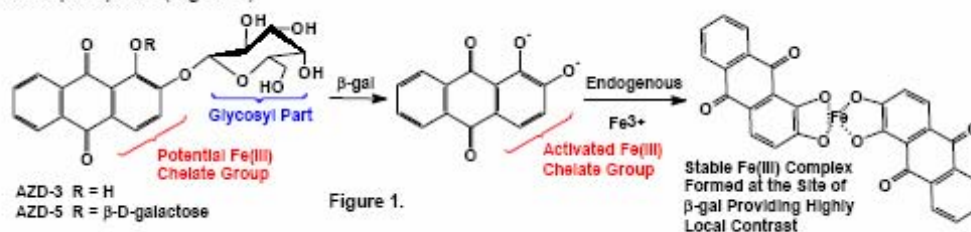
## Novel "Smart" <sup>1</sup>H MRI Contrast Agents for Assessing LacZ Gene Expression

J-X. Yu<sup>1</sup>, L. Liu<sup>1</sup>, V. D. Kodibagkar<sup>2</sup>, W. Cui<sup>1</sup>, R. D. Gerard<sup>3</sup>, R. P. Mason<sup>1</sup>

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### Introduction

The application of reporter genes to study gene expression and regulation in biological systems is common practice. Among the widely used reporter proteins,  $\beta$ -gal (*lacZ*) is recognized as the most attractive reporter gene, and its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction. Many colorimetric substrates are available commercially, but *in vivo* assays would be more powerful. Recently, Weissleder *et al.*<sup>[1]</sup> presented a near infrared *in vivo* approach based on DDAOG, Meade *et al.*<sup>[2]</sup> reported a proton MRI approach using EgadMe, and Mason *et al.*<sup>[3,4]</sup> presented both proton and <sup>19</sup>F NMR methods using S-gal<sup>TM</sup> and fluorophenol  $\beta$ -D-galactosides. S-gal<sup>TM</sup> was effective, but the molecule was designed for histology and can be optimized for *in vivo* MRI applications. We now present analogs of S-gal<sup>TM</sup> further demonstrating this fundamentally novel mechanism of "smart" <sup>1</sup>H MRI contrast agent, whereby the paramagnetic material is not generated until  $\beta$ -gal acts on the substrates (here AZD-3 or AZD-5) in the presence of Fe<sup>3+</sup> ions to generate a precipitate (Figure 1).



### Materials and Methods

AZD-3 and AZD-5 were stereoselectively synthesized and characterized in our lab. MR images were obtained using a Varian Unity INOVA 400 NMR spectrometer with gradient echo imaging: TR=100ms, Flip angle=10°, TE=multiple values 3-30ms, Matrix=256×128, FOV=48×24mm. As an example 10<sup>5</sup> PC3-LacZ or wild type cells were layered in agarose ferric ammonium citrate (2.5  $\mu$ g/mL) and AZD-5 (1.5  $\mu$ g/mL).

### Results

A series of tests in solution and cultured tumor cells proved the principle. Both AZD-3 and AZD-5 were cleaved effectively by  $\beta$ -gal generating an intense black precipitate, which provides strong T<sub>2</sub><sup>\*</sup> relaxation and intense Fe(III)-based <sup>1</sup>H MRI contrast (Figure 2).

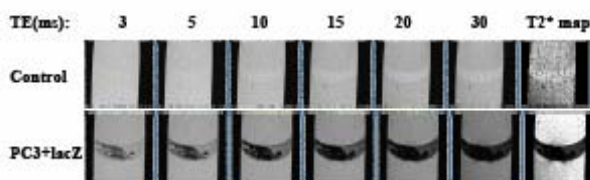
### Conclusion

These results provide further evidence for the broad specificity of  $\beta$ -gal to cleave diverse substrates. The black paramagnetic precipitate is analogous to that formed using commercial S-gal<sup>TM</sup> and demonstrates the potential for derivatizing the substrate to optimize the MR active molecule. Here, ferric ions were added. However, it is noteworthy that tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilization of iron and thus endogenous ferric ions may suffice for *in vivo* applications. We believe, this novel "smart" Fe(III)-based <sup>1</sup>H MRI contrast agent mechanism holds great promise as a fundamentally different <sup>1</sup>H MRI platform for *in vivo* assessing *lacZ* gene activity.

Supported by Cancer Imaging Program P20 CA086354 and BTRP P41RR02584.

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- [2] Louie AY, Meade TJ *et al.*, *Nature Biotechnol.*, **2000**, *18*, 321-325.
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ISMRM 13th Scientific Meeting in Miami Beach, Florida, USA, May 2005

**<sup>19</sup>F CSI of gene-reporter molecule OFPNPG**

**Vikram Kodibagkar<sup>1</sup>, Jianxin Yu<sup>1</sup>, Li Liu<sup>1</sup>, Steven Brown<sup>2</sup>, Hoby P. Hetherington<sup>3</sup>, Robert Gerard<sup>4</sup>, and Ralph P. Mason<sup>1</sup>**

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**Synopsis:** The *lacZ* gene, encoding the enzyme  $\beta$ -galactosidase ( $\beta$ -gal) was historically the most attractive reporter gene for molecular biology. 2-Fluoro-4-nitrophenol- $\beta$ -D-galactopyranoside (OFPNPG) belongs to a novel class of NMR active molecules (fluorophenyl- $\beta$ -D-galactopyranosides), which are highly responsive to the action of  $\beta$ -gal. OFPNPG has a single <sup>19</sup>F peak with chemical shift of 55 ppm. It is cleaved by  $\beta$ -gal to OFPNP, which has a pH sensitive chemical shift of 59-61 ppm. The large change in the chemical shift allows us to image  $\beta$ -gal activity with magnetic resonance chemical shift imaging (CSI). We will present the results of <sup>19</sup>F CSI studies of enzyme activity and *lacZ* gene expression in 9L glioma and PC3 cells. Our results indicate that OFPNPG is a promising gene-reporter molecule for future *in vivo* studies.

ISMRM 13th Scientific Meeting in Miami Beach, Florida, USA, May 2005